Folate Receptor Autoantibodies Are Prevalent in Children Diagnosed with Autism Spectrum Disorder, Their Normal Siblings and Parents

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Folate deficiency can affect fetal and neonatal brain development Considering the reported association of Folate receptor alpha (FRα) autoantibodies (Abs) with autism and developmental disorders, we sought to confirm this in families of 82 children with ASD, 53 unaffected siblings, 65 fathers, and 70 mothers, along with 52 unrelated normal controls. Overall, 76% of the affected children, 75% of the unaffected siblings, 69% of fathers and 59% of mothers were positive for either blocking or binding Ab, whereas the prevalence of this Ab in the normal controls was 29%. The Ab was highly prevalent in affected families including unaffected siblings. The appearance of these antibodies may have a familial origin but the risk of developing ASD is likely influenced by other mitigating factors since some siblings who had the antibodies were not affected. The antibody response appears heritable with the blocking autoantibody in the parents and affected child increasing the risk of ASD. ***Autism Res*** *2018, 0: 000–000.* © 2018 International Society for Autism Research, Wiley Periodicals, Inc.

Lay Summary: Folate is an essential nutrient during fetal and infant development. Autoantibodies against the folate receptor alpha can block folate transport from the mother to the fetus and to the brain in infants. Children diagnosed with autism and their immediate family members were evaluated for the prevalence of folate receptor autoantibodies. The autoantibody was highly prevalent in affected families with similar distribution in parents, normal siblings and affected children. The presence of these antibodies appears to have a familial origin and may contribute to developmental deficits when combined with other factors.

Keywords: autism; folate receptor; autoantibodies

Introduction

Autism spectrum disorders (ASD) are a group of developmental disorders characterized by atypical socialization and communication along with repetitive and ritualistic behaviors. The clinical phenotype can vary with no specific underlying causally-related treatment. Behavioral intervention has been helpful in increasing social communication skills and antipsychotic drugs have been used to decrease problem behaviors [Johnson & Myers 2007; Loth, Murphy, & Spooren, 2016]. Multiple gene variants have been identified in ASD and therefore no single gene defect can be uniquely linked to the ASD phenotype [De Rubeis & Buxbaum, 2015; Huguet, Benabou, & Bourgeron, 2016]. Folate receptor alpha autoantibodies (FRαAbs) were first described in mothers with a history of neural tube defect (NTD) pregnancy and provided an explanation for the pathology in the fetus in the absence of folate deficiency in the mother. These Abs can block folate transport from the mother to the fetus [Rothenberg et al., 2004]. Subsequently, these autoantibodies were identified in children with cerebral folate deficiency syndrome (CFDS) and the low CSF folate in these patients was attributed to the antibodies blocking folate transport across the choroid plexus [Ramaekers et al., 2005].

In the rat model, exposure to rat FRα polyclonal antibodies during gestation and the pre-weaning period has produced severe behavioral deficits similar to those seen in ASD [Sequeira et al., 2016; Desai et al., 2016a]. FRα is highly expressed in the placenta and in the choroid plexus to transport folate to the fetus and to the brain respectively. Blocking or binding Abs against the FRα can disrupt folate transport. Children with CFDS develop progressive developmental deficits in the form of unrest, irritability, insomnia, hypotonia, ataxia, delayed speech and dyskinesias. A consistent biochemical abnormality observed in these children was a low

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CSF folate concentration. Many of their clinical features overlap with those in ASD and these children respond well to high-dose folinic acid treatment, especially if the diagnosis is made early in infancy [Ramaekers, Sequeira, & Quadros, 2013a]. Recent studies have shown a significant association of FRαAbs with ASD in children and their parents [Ramaekers, Quadros, & Sequeira, 2013b; Frye et al., 2013, 2016]. The present study extends these initial observations to families with a child having ASD.

Study Design and Analyses

Study subjects and family members were recruited as part of an extended study of ASD at the Institute for Basic Research in Developmental Disabilities (IBR), Staten Island, New York between the years 2000 and 2017. The 82 children diagnosed with ASD, pervasive developmental disorder (PDD) -not otherwise specified, childhood disintegrative disorder, or Asperger's disorder per Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV or DSM-IV-TR criteria, consisted of 65 boys and 17 girls ranging in age from 1.6 to 15 years. Three pairs of siblings occurred among the patients. Among the affected siblings, 15 (18%) were first born with additional siblings and 38 (46%) were the only child. In addition, 53 unaffected siblings (22 brothers; 29 sisters, and one half-brother and one half-sister) were tested, as were 70 mothers and 65 fathers. Unrelated, non-ASD controls comprising 24 boys and 28 girls, aged 3 to 17 years were recruited from the pediatric practice of one of the authors (CM). The demographic and clinical details of these subjects are provided in Table 1.

Diagnosis of ASD was established using parent interview (including the Autism Diagnostic Interview, Revised [Lord, Rutter, & Le Couteur, 1994] in 54 cases, using AGRE diagnostic criteria: https://research.agre.org/program/diag.cfm.), the Autism Diagnostic Observation Schedule-Generic (ADOS-G), and the PDD Behavior Inventory (PDDBI) [Lord et al., 2000; Cohen et al., 2010]. The Vineland Adaptive Behavior Scales were used for assessing adaptive functioning [Sparrow, Balla & Cicchetti, 1984; Sparrow, Cicchetti & Balla, 2005]. The diagnostic assessment by the clinical research staff was conducted blinded to FRαAb status. All families had consented or were reconsented for this study. Approval was obtained from the Institutional Review Boards and Institutional Biosafety Committees

De-identified and coded serum samples sent from IBR to SUNY, were assayed for blocking Abs by an *in vitro* functional blocking assay and an enzyme-linked immunosorbant assay (ELISA) specific for binding IgG anti-FRαAbs [Sequeira, Ramaekers, & Quadros, 2013].

Statistical Analysis: Analytic techniques included unpaired t-tests, logistic regressions, and Fisher's exact test. Analyses were performed in version 15.0 of Stata (StataCorp, College Station, TX, USA, 2017).

Results

The blocking Ab titer in affected children, unaffected siblings and parents ranged from 0.97 to 14.27 pmoles/ml serum and the binding Ab titer in these groups varied between 0.21 and 1.95 pmoles IgG/ml serum. Only two control subjects had the blocking Ab (1.66 and 2.24 pmoles) and 14 of the 52 controls were positive for the binding Ab with a titer of 0.21 to 0.98 pmoles/ml serum. The prevalence and mean levels of FRα blocking and binding Abs among affected children, unaffected siblings, parents, and unrelated controls are shown in Table 2. The Abs were highly prevalent in affected families, with a large proportion of unaffected siblings also positive for the Abs. Overall, 62 of the 82 affected siblings, 40 of the 53 unaffected siblings, 45 of the 65 fathers, and 41 of the 70 mothers were positive for either blocking or binding Ab, yielding an overall prevalence among patients and first-degree relatives of 69.6%. Overall, 76% of the affected and unaffected siblings and 59–69% of the parents were positive for either blocking or binding Ab. By contrast, 15 of the 52 normal controls (29%) were positive for either blocking or binding Ab ($P < 0.001$, Fisher's exact test). (Fig. 1).

Thus, among affected siblings the presence of Abs was strongly associated with ASD status (O.R.7.65, 95% C.I. 3.49, 16.74; $P < 0.0001$) compared to unrelated controls. Occurrence of the blocking and binding Abs were not significantly associated with each other, Fisher's exact test); only 14 ASD subjects and one control had both. Presence of the blocking Ab by itself had an O.R. of 17.19 (95% C.I. 3.91, 75.60; $P < 0.0001$), while the binding Ab alone had an O.R. of 3.14 (95% C.I. 1.48, 6.66; $P < 0.002$). Modeled together, the blocking Ab maintained an O.R. 19.25 (95% C.I. 4.27, 86.82, $P < 0.0001$) while the binding Abs O.R. was 3.56 (95% C.I. 1.57, 8.10, $P = 0.002$)), indicating that each form of Ab conferred independent risk. Addition of an interaction term to the model to determine the additive effect of both types of antibodies compared to each type of antibody alone, resulted in no significant change in fit. The mean blocking and binding titers among all affected and unaffected children with the respective antibodies (blocking, 7.39 and 6.39 respectively; binding, 0.51 for each group) did not differ significantly. In one set of triplets, where one of the triplets and an older brother were affected only the father was positive for the binding Ab. There were three sets of twins in

Table 1. Demographics and Clinical Characteristics of Participants

(A) Demographics and characteristics of participants

Participants	Total (*N*)	Male (*N*)	Female (*N*)	Age (years) Mean (SD)
Affected children	82	65	17	5.18 (2.61)
Unaffected siblings	53	23	30	6.73 (4.20)
Parent-mother	70	–	70	Adult
Parent-father	65	65	–	Adult
Controls	52	24	28	11.18 (3.98)
(B) Total clinical characteristics of affected children (*N* = 78) and siblings (*N* = 4)				
Diagnosis	**Total male and female**	**Male**	**Female**	
Autistic disorder	66	55	11	
PDD-NOS[a]	13	9	4	
Disintegrative disorder	1	1	0	
Asperger's disorder	2	2	0	
ADI-R/AGRE criteria[b]	**Total male and female (*N* = 54)[b]**	**Male**	**Female**	
Autism	48	38	10	
Not quite autism	3	3	0	
Broad spectrum	3	2	1	
ADOS-G criteria	**Total male and female (*N* = 66)[c]**	**Male**	**Female**	
Autism	62	51	11	
Spectrum	4	3	1	
(C) Vineland adaptive behavior scale domains [mean (SD)]				
Domain	**Male (*N* = 65)**	**Female (*N* = 15)**		
Communication	66 (24)	68 (17)		
Daily living skills	60 (16)	60 (13)		
Socialization	63 (15)	62 (9)		
(D) PDDBI domain & autism composite T-scores[e] [ASD expected mean (SD) = 50 (10)]				
Domain	**Male**	**Female**		
Approach-withdrawal problems				
SENSORY[d]	50 (9)	45 (9)		
RITUAL	47 (9)	49 (8)		
SOCPP	51 (9)	50 (10)		
SEMPP	49 (11)	49 (9)		
AROUSE	51 (10)	46 (10)		
FEARS	48 (9)	48 (11)		
AGG	49 (9)	47 (13)		
Receptive/expressive social communication abilities				
SOCAPP	48 (9)	51 (10)		
EXPRESS	49 (10)	49 (9)		
LMRL	48 (10)	52 (8)		
Autism composite				
AUTISM/C	50 (10)	49 (8)		

[a] Pervasive developmental disorder-not otherwise specified (includes one female with Rett syndrome features).

[b] From https://research.agre.org/program/diag.cfm. *Autism* is identified using the well-validated ADI-R scoring algorithm. *NQA (Not Quite Autism)* represents individuals who are no more than one point away from meeting the autism cutoffs on any or all of the three "content" domains (i.e., social, communication, and/or repetitive behaviors), plus meeting the autism cutoff on the "age of onset" domain; or, individuals who meet the autism cutoffs on all three "content" domains, but do not meet the autism cutoff on the "age of onset" domain. *Broad spectrum* defines individuals who show symptoms along the spectrum of pervasive developmental disorders. This is a broad diagnostic category that encompasses individuals ranging from mildly- to severely impaired. This category potentially includes such pervasive developmental disorders as PDD-NOS and Asperger's disorder, which are used in many genome scans; however, this classification is not based on any validated algorithms and may include individuals who do not meet full criteria for any DSM-IV pervasive developmental disorder."

[c] Completed where age and communication age appropriate and when reliability training had been completed.

[d] $t(77) = 2.02$, $P < 0.05$; all other T-score means do not show a sex difference. All means are at the expected level for children diagnosed with ASD.

[e] PDD Behavior Inventory (PDDBI) Scoring System (T-Scores): Each domain and composite were age-normed and per a T-score (mean = 50; SD = 10) system. Please note that the higher the T-scores for the "Approach-Withdrawal" domains and the Autism Composite Score, the more "severe" or discrepant that child's scores are from the average child with autism; the higher the T-score for the "Receptive/Expressive Social Communication Abilities" domains, the better that child's skills are relative to the average child with autism (see Cohen et al., 2010) for more information on the PDDBI domains.

Key to abbreviations used in table derived from The PDD Behavior Inventory (PDDBI): *Abbreviations*: SENSORY, sensory/perceptual approach behaviors; RITUAL, ritualisms/resistance to change; SOCPP, social pragmatic problems; SEMPP, semantic/pragmatic problems; AROUSE, arousal regulation problems; FEARS, Specific fears; AGG, aggressiveness; SOCAPP, social approach behaviors; EXPRESS, expressive language, LMRL, learning, memory, and receptive language; AUTISM/C, autism composite.

Table 2. Percentage Occurrence and Mean Levels of Blocking and Binding Autoantibodies in Study Subjects

	Blocking antibodies			Binding antibodies		
	N	Percent	Pmol/ml (mean, SD)	*N*	Percent	Pmol/ml (mean, SD)
Affected children	81	40.7%	3.01 (4.95)	82	53.7%	0.272 (0.320)
Females	17	52.9%	3.99 (5.69)	17	52.9%	0.308 (0.332)
Males	64	37.5%	2.75 (4.75)	65	53.8%	0.262 (0.319)
Parents	135	37.8%	1.32 (2.64)	135	40.0%	0.208 (0.335)
Mothers	70	31.4%	0.94 (1.97)	70	35.7%	0.212 (0.384)
Fathers	65	44.6%	1.72 (3.18)	65	44.6%	0.204 (0.276)
Unaffecetd Siblings	53	43.4%	2.78 (4.89)	53	54.7%	0.278 (0.345)
Sisters	30	40.0%	3.40 (5.56)	30	63.3%	0.281 (0.256)
Brothers	23	47.8%	1.97 (3.81)	23	43.5%	0.274 (0.441)
Controls	52	3.8%	0.08 (0.38)	52	26.9%	0.109 (0.213)
Females	28	3.6%	0.08 (0.42)	28	21.4%	0.119 (0.260)
Males	24	4.2%	0.07 (0.34)	24	33.3%	0.098 (0.145)

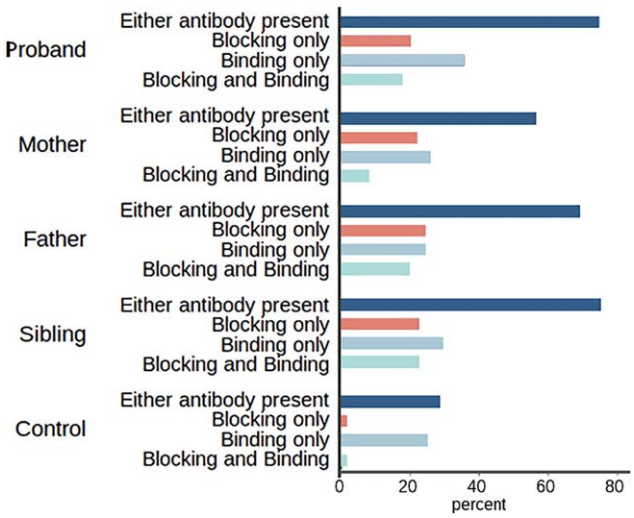

Figure 1. The prevalence of folate receptor autoantibodies in families with an affected child and in controls.

the study and all were affected with FRα autoantibody present in the affected twins and their parents.

Heritability of FRαAbs: Among 48 affected children whose mothers did not have the blocking antibody, 13 (27%) had the Ab and among 29 whose mothers had the Ab, 19 (66%) had the antibody, yielding an O.R. of 5.12 (95% C.I. 1.89, 13.85; $P = 0.002$), Fisher's exact test). The presence of the blocking Ab in the father, on the other hand, did not confer significantly increased likelihood of the presence of the blocking Ab in the child: 12 of 37 affected children (32%) whose father did not have the Ab and 19 of 36 affected children (53%) whose father had the Ab had it themselves. Modeling only the blocking Ab on both parents' Ab status yielded a significant effect for mother's Ab status ($P = 0.001$) but not for father's Ab status.

Among 33 unaffected children whose mothers did not have the blocking antibody, 10 (30%) had the Ab and among 14 whose mothers had the Ab, 12 (86%) had the antibody, yielding an O.R. of 13.80 (95% C.I. 2.59, 73.38; $P = 0.001$), Fisher's exact test). The presence of the blocking Ab in the father, on the other hand, conferred a lower risk of the presence of the blocking Ab in the unaffected child: 7 of 24 unaffected children (29%) whose father did not have the Ab and 14 of 23 unaffected children (61%) whose father had the Ab had it themselves, yielding an O.R. of 3.78 (95% C.I. 1.12, 12.73; $P = 0.041$), Fisher's exact test). Modeling only the blocking Ab on both parents' Ab status yielded a significant effect for mother's Ab status ($P = 0.003$) but not for father's Ab status.

Among 81 children whose mothers did not have the blocking antibody, 23 (28%) had the Ab and among 43 whose mothers had the Ab, 31 (72%) had the antibody, yielding an O.R. of 6.51 (95% C.I. 2.86, 14.83; $P < 0.0001$), Fisher's exact test). The presence of the blocking Ab in the father, on the other hand, conferred a lower risk of the presence of the blocking Ab in the child: 19 of 61 children (31%) whose father did not have the Ab and 33 of 59 children (56%) whose father had the Ab had it themselves, yielding an O.R. of 2.80 (95% C.I. 1.33, 5.92; $P = 0.010$), Fisher's exact test). Modeling only the blocking Ab on both parents' Ab status yielded a significant effect for mother's Ab status ($P < 0.001$) but not for father's Ab status.

The highest percentage of affected siblings was observed when both parents were positive for the Ab. This distribution decreased if the father or mother was positive or if both were negative (Table 3A). When unaffected siblings were added to this distribution, highest percentage of affected siblings were observed when mother, father and the unaffected siblings were positive for the antibody (Table 3B).

Discussion

Folate deficiency has a direct effect on fundamental processes of cellular replication and therefore plays an

Table 3. Binding or Blocking FR Autoantibody Distribution in Families

(A) Families grouped according to the presence or absence of antibodies among proband, mother, and father

Group	*Proband*	*Mother*	*Father*	*Frequency*	*Percent*
1	yes	yes	yes	25	32.1
2	yes	yes	no	11	14.1
3	yes	no	yes	13	16.7
4	yes	no	no	9	11.5
5	no	yes	yes	5	6.4
6	no	no	yes	6	7.7
7	no	yes	no	4	5.1
8	no	no	no	5	6.4

(B) Families grouped according to the presence or absence of antibodies among proband, siblings, mother and father

Group	Proband	Siblings	Mother	Father	Frequency	*Percent*
1	yes	yes	yes	yes	16	20.5
2	yes	no	yes	yes	9	11.5
3	yes	yes	no	yes	8	10.3
4	yes	no	yes	no	8	10.3
5	yes	no	no	yes	5	6.4
6	yes	no	no	no	7	8.9
7	yes	yes	yes	no	3	3.9
8	no	no	no	yes	5	6.4
9	no	no	yes	no	4	5.1
10	no	yes	yes	yes	4	5.1
11	yes	yes	no	no	2	2.6
12	no	yes	no	yes	1	1.3
13	no	yes	no	no	1	1.3
14	no	no	yes	yes	1	1.3
15	no	yes	yes	no	0	2.6
16	no	no	no	no	4	5.1

important role in fertility, pregnancy, fetal development and ultimately in the development and function of the brain [Desai et al., 2016b]. Extensive research to identify the cause(s) of ASD has not yielded definitive answers. The heritability of ASD is approximately 55% and the majority of genetic factors are considered to be due to common variants [DeRubeis et al., 2015]. Evidence from biochemical, genetic and epidemiologic studies suggests multiple factors and alterations in multiple genes associated with the disorder [Gaugler et al., 2014]. While environmental insults and specific gene defects can potentially contribute to developmental disorders, folate, by virtue of its involvement in multiple metabolic pathways including purine and pyrimidine synthesis, its role in epigenetic modifications and gene expression profiles, can have profound effects on the development and functional refinement of the brain.

Disruption of folate transport by an Ab directed at the primary route of folate uptake into the brain coupled with the potential for inflammation and tissue damage can further affect structure and function [Desai et al., 2016]. The association of low CSF folate and FRαAbs in CFDS [Ramaekers et al., 2005] and the significant association of FRαAbs with ASD [Frye et al., 2013] provide compelling justification to further investigate the role of these antibodies in fetal and neonatal brain development and function. A previous study examining the prevalence of FRαAbs in parents of children with ASD showed that about 26% of the mothers and 18% of the fathers were positive for the autoantibody compared to only 3% of parents with normal children [Ramaekers et al., 2013b]. Siblings were not evaluated in this study. The present study indicates that the autoantibody is highly prevalent and equally distributed in families and not restricted to the affected child or mother. This negates a direct role of FRαAbs in the pathology of developmental disorders but does not preclude it as a contributing factor. Maternal antibody titer and folate status during pregnancy and a similar disposition in the infant could influence the outcome. It is likely that the pathologic effects of the Abs are exerted early on during fetal development and during early infancy and the outcome may be dictated by many factors; among them maternal Ab titer and folate status during pregnancy and the age at which Abs appear in the child. In addition to fetal exposure to maternal Abs, paternal FRαAbs could exert their influence on imprinting genes by epigenetic modifications. This raises the possibility of supplementing the parents positive for the FRαAbs before pregnancy occurs and the mother throughout the pregnancy with folinic acid to prevent folate deficiency in the fetus. Therefore, identification of parents and infants with the Abs, provides the potential for early intervention to prevent or reduce the risk of ASD in the offspring.

While folic acid and N^5-methyl folate are actively transported by FRα, folinic acid, a reduced form of folate, is transported via a different mechanism involving the reduced folate carrier. Treating children with folinic acid has shown some improvement in neurologic deficits especially in younger children and improvement in language and communication in older children [Frye et al., 2013, 2016]. Although, at present, the clinical trials using folinic acid have some limitations, the consistent positive findings of this safe and well-tolerated treatment raises the potential of a treatment that addresses core symptoms of ASD while also targeting pathophysiological abnormalities.

Evidence in support of the pathologic effects of the Abs has been recently shown in a rat model. Pups exposed to rat anti FRα polyclonal antibodies *in utero* and during the pre-weaning period, show severe learning and behavioral deficits [Sequeira et al., 2016]. Administration of folinic acid alone or in combination with dexamethasone to the pregnant dam at the time of Ab exposure, protected the offspring from the pathologic effects of the Ab [Desai et al., 2016a]. These observations are strong evidence of intervention strategies

that could potentially work in humans. The prevalence of Abs in one or both parents and in unaffected siblings suggests a familial origin of the antibody response. Even though this study fails to establish a direct association of FRαAbs with ASD, the high prevalence of the autoantibody in affected families provides a marker for increased risk of developmental disorders. The heritability of the disorder and multiple genes/pathways that are affected in ASD, suggests genetic and epigenetic alterations contributing to the genotype and the phenotype influenced by the folate status. Intervention strategies in parents and children to reduce the autoantibody titer and treating with folinic acid may be a strategy to prevent and treat developmental deficits associated with the presence of FRα antibodies.

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Conflict of interest

Two of the authors (JMS and EVQ) are inventors on a US patent for the detection of FRalpha autoantibodies issued to the Research Foundation of the State University of New York, USA. All other authors declare no conflict of interest.

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